Angiotensin II type 2 receptor gene polymorphisms in cardiovascular disease

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*J Renin Angiotensin Aldosterone Syst* 2010; 11; 79 originally published online Oct 27, 2009;
DOI: 10.1177/1470320309347782

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Abstract
Considerable progress in our understanding of the role of the angiotensin II type 2 (AT₂) receptor in the development of cardiac hypertrophy and coronary artery disease has been achieved using in vitro and in vivo animal models. Our understanding in humans, however, has been hindered by the lack of availability of specific AT₂ receptor agonists and antagonists suitable for human study. Nevertheless, an alternative approach involving genotyping humans for a functional polymorphism within the AT₂ receptor gene (–1332G/A) has been used in several association studies to elucidate the pathogenic role of the AT₂ receptor in cardiovascular disease. Both the A allele and the G allele have independently been associated with left ventricular remodelling. However, the methods of measuring left ventricular mass, sodium balance, age and degree of remodelling appear to influence the outcome. An association of carriers of the G allele and premature coronary artery disease has also been established, particularly in males presenting with stenotic atherosclerosis requiring revascularisation. However, the methods of measuring left ventricular mass, sodium balance, age and degree of remodelling appear to influence the outcome. An association of carriers of the G allele and premature coronary artery disease has also been established, particularly in males presenting with stenotic atherosclerosis requiring revascularisation. At the molecular level, it remains unclear as to whether carriers of the G allele express more or fewer AT₂ receptors when compared to carriers of the A allele. Consequently, it is presently not possible to definitively interpret the role of the AT₂ receptor in human cardiovascular disease from these association studies.

Introduction
Angiotensin II (Ang II) is an octapeptide hormone which mediates the main effects of the renin-angiotensin-aldosterone system through the binding and activation of two specific Ang II receptors – type 1 (AT₁) and type 2 (AT₂). The vast majority of the known physiological actions of Ang II are mediated by AT₁ receptors, including vasoconstriction, aldosterone secretion, antinaturesis, increased cardiac contractility, cell proliferation, hypertrophy, as well as pro-oxidative and pro-inflammatory effects. Longer term, activation of AT₁ receptors is believed to lead to pathophysiological conditions such as hypertension, cardiac and vascular hypertrophy, fibrosis, atherosclerosis and myocardial infarction. In contrast to the AT₁ receptor, the actions mediated by activation of the AT₂ receptor are much less well understood.

AT₁ receptors are expressed at high levels in foetal tissues, but their expression decreases after birth to a comparatively low level in the adult, being restricted to a few organs such as specific regions within the brain, adrenal glands, heart, kidney, vascular endothelium, myometrium and ovary. AT₂ receptor expression, however, has been observed to increase under pathological conditions including hypertension, vascular injury, myocardial infarction, heart failure and stroke. Knowledge of the cell signalling and physiological actions of the AT₂ receptor has continued to develop since the mid-1990s. It is generally agreed that the AT₂ receptor opposes many of the effects of the AT₁ receptor by inducing vasodilatation, naturesis, inhibiting cell proliferation and stimulating apoptosis.

Interest in the association of AT₂ receptor gene polymorphisms and cardiovascular disease first gained attention following the reporting of a genome-wide linkage scan of early- and late-settlement populations of Finland for premature coronary heart disease (CHD). Two loci, on chromosomes 2 and X, were linked to premature CHD in both separate and combined data analysis. The identification of the X locus (Xq23-26) was particularly interesting as the majority of the linkage information in this study was derived from males, and males have a higher frequency of premature CHD. As males are hemizygotes for disease alleles on chromosome X, they have a higher probability to show the effects of recessively-acting X-chromosomal susceptibility genes than do women. Examination
of the X locus for candidate genes underlying CHD identified one interesting candidate, the AT2 receptor.

The gene structure of the human AT2 receptor, which spans approximately 5 kb, was first elucidated in 1995. The gene consists of three exons interspaced by two introns. Regulatory elements are located in the first intron in addition to the promoter region, whilst the third exon contains the complete protein-coding sequence which translates into a 363-amino acid G-protein-coupled receptor. A number of polymorphic sites, at both high and very low frequency, have been identified throughout the AT2 receptor gene. This review focuses primarily on the common polymorphism –1332G/A (relative to the start codon in exon three; rs1403543), also known as 1675G/A (relative to the transcription start), but also briefly mentions 3123C/A (located in the 3'-UTR). Recent haplotype analysis of the AT2 receptor gene has revealed that four common haplotypes account for over 90% of the haplotypes present in a Caucasian population. Furthermore, the –1332G/A and 3123C/A polymorphisms are in high linkage disequilibrium.

**In vitro characterisation of the –1332G/A polymorphism**

The –1332G/A polymorphism was first described during an investigation into the role of the AT2 receptor gene in congenital anomalies of the kidney and urinary tract (CAKUT). Although low in frequency, predominantly male AT2 receptor gene-knockout mice were observed to display phenotypes that resembled human CAKUT. This led the investigators to screen for polymorphisms within the human AT2 receptor gene, resulting in the identification of a common A to G transition in intron 1 at position –1332. Comparisons of CAKUT cases and controls in both male Caucasians of American (13 patients/31 controls) and German (23 patients/24 controls) origin revealed an excess of the G allele in the disease groups. It should be noted that this association with CAKUT has not been confirmed in later studies.

Sequence analysis revealed that the polymorphism was within the only 7-nucleotide sequence that conforms to the consensus for the DNA sequence lariat branchpoint in intron 1, important for mRNA processing. This raised the question as to whether this polymorphism might alter the AT2 receptor mRNA splice pattern and hence downstream expression levels of functional AT2 receptors. AT2 receptor mRNA isolated from fibroblast cell lines derived from hemizygous males and analysed by RT-PCR revealed genotype-dependent differences in the AT2 receptor splice pattern. Carriers of the A allele only expressed mRNA containing exons 1, 2 and 3 (Ex1/2/3), whilst G allele carriers only expressed mRNA containing exons 1 and 3 (Ex1/3), exon 2 being selectively omitted. Furthermore, the G allele carriers expressed markedly lower levels of AT2 receptor mRNA. This abnormal and inefficient splicing of pre-mRNA from the G allele was verified when the AT2 receptor transcripts from freshly harvested homozgyous female uterine samples were examined using an RNase protection assay. The authors concluded that the –1332G/A polymorphism was functional, with carriers of the G allele predicted to be associated with reduced AT2 receptor expression.

In the same year, the two splice variants of the AT2 receptor, Ex1/2/3 and Ex1/3, were also identified and reported in normal and failing human hearts. However, in these studies, all five hearts expressed both transcripts, with transcript Ex1/2/3 constituting 92% and transcript Ex1/3 8% of all AT2 receptor mRNAs in both the ventricles and atria. Furthermore, using a luciferase reporter gene construct containing the proximal AT2 receptor promoter and the 5'-UTR of either cDNA Ex1/2/3 or cDNA Ex1/3, revealed that the former construct expressed 31% lower luciferase activity. It was concluded that transcriptional regulation by differential splicing of the AT2 receptor mRNA might modulate AT2 receptor expression.

An alternative interpretation of the functional significance of the –1332G/A polymorphism originally proposed by Nishimura and colleagues was reported in 2005. Genotyping of myocardial tissue previously reported to express both Ex1/2/3 (92%) and Ex1/3 (8%) AT2 receptor splice variants revealed that the myocardial AT2 receptor mRNA splice pattern was not detectably affected by the –1332 genotype. Further confirmation of these findings was obtained through transient transfection of human HT1080, rat p12W and rat vascular smooth muscle cells with an AT2 receptor minigene/luciferase reporter construct. The construct consisted of a genomic AT2 receptor fragment of either allele comprising the core promoter, exons 1 and 2, introns 1 and 2, and exon 3 up to the translation start codon. Subsequent RNA analysis of these transfected cells revealed the same AT2 receptor mRNA splice patterns as the myocardial tissue samples. However, in contrast to the identical mRNA splice patterns, luciferase activities driven by the two AT2 receptor minigene/luciferase reporter constructs were significantly different. Luciferase activity in cells transfected with the
G allele construct was 26% higher in human HT1080, 38% higher in rat PC12W and 55% higher in rat vascular smooth muscle cells compared to those expressed by the A allele construct. The authors suggested that the luciferase levels might be considered as representative of AT1 receptor protein levels in vivo and thus that carriers of the G allele would express higher levels of AT1 receptors relative to carriers of the A allele. It is important to note, however, that no definitive mechanism underlying this difference was reported, although the possibility that a slightly higher affinity of the splicing factors to the G allele which might result in a more efficient pre-mRNA processing and thereby protein expression was proposed.

The role of the AT1 receptor in cardiac hypertrophy

The potential differences in receptor expression driven by the A and G alleles might be particularly important in the development of cardiac hypertrophy and coronary artery disease (CAD) where AT1 receptor expression is known to increase. Furthermore, until recently, no AT1 receptor agonists or antagonists were available for human use to elucidate the physiological and pathophysiological role of the AT1 receptor. Thus, by studying the relationship between the genotypes of this AT1 receptor polymorphism and the above conditions it might be possible to elucidate the role of the AT1 receptor in the development and/or progression of cardiovascular disease in humans.

In animal studies, the role of the AT1 receptor in the development of cardiac hypertrophy is controversial. In neonatal rat cardiomyocytes, AT1 receptor stimulation inhibits the growth of both cardiac myocytes and fibroblasts by counteracting AT1 receptor signalling. However, overexpression of AT1 receptors in isolated rat cardiomyocytes does not prevent AT1 receptor-mediated myocyte hypertrophy. Some studies in AT1 receptor-knockout mice have reported that left ventricular hypertrophy (LVH) did not develop in response to pressure overload, to being induced by constricting the abdominal aorta, or by the infusion of Ang II, suggesting an obligatory role of the AT1 receptor in the development of cardiac hypertrophy. Others have reported that AT1 receptor-knockout mice have greater cardiac remodelling/hypertrophy after myocardial infarction compared with wild-type mice, implying the AT1 receptor is protective. It has also been reported that overexpressing AT1 receptors in mice, specifically in cardiomyocytes, had no effect on the development of Ang II-induced cardiomyocyte hypertrophy, whilst others have reported that AT1 receptor gene transfer attenuated cardiac hypertrophy and fibrosis in spontaneously hypertensive rats. At least part of this inconsistency relates to the experimental approaches used, mainly in vivo studies using wild-type and AT1 receptor-transgenic or -knockout animals. These contradictions might be related to differences in strains (genetic background) of animals, compounded by other cellular, circulating and paracrine factors which might modulate the AT1 receptor phenotype observed.

Association studies of the -1332G/A polymorphism and left ventricular remodelling

The first study to investigate the association of the human AT1 receptor polymorphism and left ventricular remodelling was undertaken with a male Caucasian cohort selected from students at a Bavarian university. The study consisted of 60 normotensives and 60 untreated subjects with mildly elevated blood pressure (BP of ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic) aged between 20 and 40 years old (mean age 26±3 years). Two-dimensional guided M-mode echocardiography was used to measure left ventricular wall thicknesses. The main finding of the study was that, unlike the normotensive subjects, carriers of the A allele in the mildly elevated BP group were associated with a higher left ventricular mass, relative to carriers of the G allele. Thus, it appeared that the AT1 receptor genotype was associated with left ventricular structural adaptive processes in hypertensive subjects prone to develop myocardial hypertrophy in response to an increased afterload. A year later, members of the same group reported on findings in two Glasgow heart studies (GLAOLD and GLAECO). In the GLAOLD study, the A allele was associated with LVH in males aged 55–74 years old and recurrent ischaemia and myocardial infarction in elderly females, lending some support to their earlier finding in young hypertensive males. However, these findings were not replicated in the GLAECO cohort even though participants were very similar between the two cohorts, and the later collected GLAECO cohort contained twice as many subjects.

In the European Project On Genes in Hypertension (EPOGH), a novel interaction was reported between sodium balance and the association of left ventricular remodelling with the AT1 receptor polymorphism. The study recruited White nuclear families consisting of at least one parent and two siblings. As in previous studies, echocardiography was used to measure left ventricular mass and participants collected a 24-hour urine sample to measure sodium excretion. Without taking sodium excretion into account, there was no difference in
the left ventricular mass index (LVMI) between carriers of the G or A allele in both men and women. However, when groups were split based on medians of sodium excretion, it was noted that there was a statistically significant interaction in men between the AT$_2$ receptor genotype and sodium excretion analysed as a continuous variable in relation to LVMI. A major observation was that the LVMI of carriers of the G allele increased with higher sodium excretion, whereas carriers of the A allele tended to decrease, but this was not statistically significant. A similar, but not statistically significant, trend was also observed in women. However, LVMI was lower in G than A allele carriers when sodium excretion was below the median. The authors concluded that the AT$_2$ receptor polymorphism independently influenced left ventricular mass and that salt intake modulated this genetic effect, the male carriers of the G allele being particularly sensitive.

This association has recently been validated in a study involving a repeat of the original Bavarian study of young Caucasian males with and without mild hypertension, but with twice as many subjects. In keeping with the earlier study, A allele carriers were associated with a higher left ventricular mass than G allele carriers in subjects with mild hypertension, and this difference was not observed in normotensive individuals. The authors then analysed the interaction of salt by dividing the AT$_2$ receptor genotypes based on median salt intake to obtain equally numbered subgroups. In keeping with the EPOGH study, left ventricular mass was higher in the G allele carriers with high salt intake than low salt intake. Although there was a clear increase in both systolic and diastolic BP associated with G allele carriers with a high salt intake, after applying covariance analysis there was still a clear trend towards an effect of dietary salt intake on left ventricular mass. Again, in keeping with the EPOGH study, there was no significant difference in the left ventricular mass between A allele carriers on high versus low salt intake, even though the high salt intake group was associated with higher systolic pressure.

There are two studies in the literature which have reported the association of AT$_2$ receptor polymorphisms with hypertension and salt sensitivity. In a study of healthy middle-aged Japanese men, carriers of the A allele of the 3123C/A polymorphism were observed to be associated with an increase in both systolic and diastolic BP with increasing daily levels of self-reported salt intake. Carriers of the C allele did not show this association. As mentioned earlier

Both the accuracy and the reproducibility of M-mode echocardiography, particularly in patients with LVH, have been debated. In a recent study, left ventricular mass was measured using cardiac magnetic resonance imaging, a more precise and reproducible technique, to investigate the association of the –1332G/A polymorphism with LVMI in patients with systemic hypertension. Initially, 60 healthy volunteers (30 male; mean age 43±12 years) were recruited to establish a normal range for cardiac magnetic resonance imaging left ventricular volumes and mass. Left ventricular mass in 197 patients (125 male) with essential hypertension (mean age 55±11 years) was measured and individuals were identified with LVH as having an elevated LVMI, based on the mean LVMI for normal volunteers plus two standard deviations. When left ventricular mass was assessed as a continuous variable, it was observed that G/GG carriers (homozygous males, homozygous females) were associated with a higher mean left ventricular mass compared to A/AA carriers. Furthermore, there was a higher prevalence of –1332 G/GG allele carriers in the hypertensive group with LVH compared to A/AA carriers. Additionally, there was a higher prevalence of –1332 G/GG allele carriers in the hypertensive group with LVH compared to the combined subjects without LVH (normal volunteers and hypertensives without LVH). There was also a trend for a higher prevalence of –1332 G/GG allele carriers in the hypertensive group with LVH compared to the hypertensives without LVH. These data suggest that the G allele was associated with hypertension-related LVH.

The above results are clearly opposed to those observed in the Bavarian cohort studies where increases in mean left ventricular mass were associated with the A allele. However, it is important
to note that the Bavarian cohort studies involved young males and relatively modest increases in left ventricular mass, whereas the above study investigated middle-aged individuals with established systemic hypertension and clinically relevant LVH measured by the more accurate magnetic resonance imaging technique. It is also worth noting that two of the previous studies identified that the mean left ventricular mass of only G allele carriers is sensitive to changes in environmental interactions (i.e. sodium balance) and appears to be associated with raised BP which may also be age related. Interestingly, in animal experiments, dietary sodium depletion enhances the expression of the AT2 receptor, and there is a report that older rats express higher levels of AT2 receptors in the vasculature and display AT2 receptor-mediated vasoconstriction.

The role of the AT2 receptor in atherosclerosis

The AT2 receptor has antiproliferative actions in both endothelial and vascular smooth muscle cells, which off-set the growth-promoting effects mediated by the AT1 receptor. Furthermore, its expression increases following vascular injury and myocardial infarction, and stimulation appears to reduce neointimal formation, cell proliferation and inflammation. More recently, studies using AT2 receptor/APOE double-knockout mice have reported that AT2 receptor expression is upregulated in atherosclerotic lesions. Both studies noted that after treatment with a high cholesterol diet, atherosclerotic changes were aggravated in the double-knockout mice, suggesting that the AT2 receptor plays a protective role in atherosclerotic lesion formation. Mechanisms proposed included attenuation of oxidative stress and the observation that in the absence of AT2 receptors there was enhanced accumulation of vascular smooth muscle cells, macrophages, inflammatory cells and collagen in the atherosclerotic lesions, implicating the AT2 receptor in the regulation of plaque cellularity and composition. Similar findings were recently reported in low-density lipoprotein receptor-deficient mice following overexpression of AT2 receptors in vascular tissue through systemic introduction of AT2 receptor cDNA delivered with adeno-associated virus type 2. These represent potential mechanisms by which a functional AT2 receptor gene polymorphism may modulate the development and complications of CAD. As a cautionary note, some cardiologists would consider an atherosclerotic plaque containing a reduced density of smooth muscle cells and a thin collagen cap more susceptible to rupture and hence thrombosis. Thus, whether the stimulation of the AT2 receptor is protective or harmful in CAD remains unresolved.

Association studies of the –1332G/A polymorphism and premature CAD

There are only three main studies that have addressed the association of the –1332G/A polymorphism with either CHD risk or prevalence of premature CAD. The earliest study, a prospective CHD gene association study, involved 2,579 unrelated White middle-aged men (mean age 56.1±3.5 years) studied over a 10-year period. CHD events, of which there were only 185, were defined as sudden cardiac death or symptomatic myocardial infarction, silent myocardial infarction, or coronary revascularisation. No association between genotypes was observed for baseline BP or indeed for survival by genotype in the 1,893 individuals normotensive at baseline. However, in the 322 patients that represented a retrospectively defined subgroup (with systolic, but not diastolic hypertension; systolic BP ≥160 mmHg), there was a tentative association with the A allele in decreased survival during follow-up. The authors concluded that their observation provided the first direct evidence of a role for the AT2 receptor in the pathogenesis of human CHD.

A recent investigation of the association of the –1332G/A polymorphism with CAD in 509 families (1,556 individuals) with a history of premature CAD has been reported. The Genetic Risk of Acute Coronary Events (GRACE) cohort is a collection of mainly sibling trios with at least one sibling affected with premature CAD (mean age 49.5±8.1 years) and additional unaffected siblings (mean age 56.2±9.5 years). In comparison with the use of unrelated cases and controls, this family approach avoids the confounding effect of genetic heterogeneity that would tend to result in a false positive finding. In contrast, the use of sibling trios would be predicted to reduce markedly the likelihood of false positive findings due to the presence of much greater genetic homogeneity as a result of shared inheritance. An additional advantage is that the structure of the families permits assessment of transmission disequilibrium of genetic loci. Using the X-linked sibling transmission/disequilibrium test (XS-TDT), both the linkage and association could be tested. The main finding was that the G allele occurred significantly more frequently with premature CAD than would be expected if the disease susceptibility locus and typed marker were unlinked. This was driven by a highly statistically significant result in hemizygous males in contrast to a negative result in dizygous females. The
XS-TDT was also used to evaluate an association, in the presence of linkage, for the –1332G/A polymorphism and a history of hypertension and found that the G allele occurred more frequently in the hemizygous males, but not dizygous females, although it did not reach statistical significance.

More recently, the number of families in the GRACE cohort (885 families, 2,662 individuals) has been extended to enable analysis of associations of the –1332G/A polymorphism with coronary atheroma sub-phenotypes. Myocardial infarction (assigned group 1) is considered to be a restrictive phenotype with a distinct clinical manifestation, plaque rupture with acute thrombotic occlusion. However, patients with stenotic CAD (assigned group 2) as demonstrated angiographically and/or the need for a revascularisation procedure are known to have experienced multiple sub-clinical plaque ruptures and erosive events. Demographics and cardiovascular risk factors were identical to the earlier study and confirmed the original observation that the G allele occurred significantly more frequently with premature CAD than would be expected if the disease susceptibility locus and typed marker were unlinked. Overall, the frequency of the G allele was found to be higher in group 2 patients as compared to group 1, who in turn were higher than that observed in the unaffected siblings. Furthermore, the XS-TDT for linkage between the AT2 receptor locus and group 2 confirmed an association in the presence of linkage for the G allele. This was driven by a highly statistically significant result in males in contrast to a negative result in females. The XS-TDT for linkage between the AT2 receptor locus and group 1 did not show an association, although there was a strong trend towards statistical significance in males, but not females. Extrapolating the in vitro data from the animal studies which suggest that the AT2 receptor may be a factor in the development and structural integrity of atheromas, it is possible that alterations in the levels of expression of functional AT2 receptors that result from the –1332G allele contribute to the development of plaques and hence also premature CAD as has been observed in these families.

Conclusions
There is still unresolved controversy regarding the role of the AT2 receptor in cardiovascular disease and hence conclusions that can be drawn from studying the –1332G/A polymorphism in association studies. In addition, data from two independent in vitro studies analysing the functional consequences of this polymorphism on receptor expression have reported polarised conclusions, one suggesting the G allele is associated with reduced receptor expression, and the other that the G allele is associated with increased receptor expression. Importantly, neither of these studies measured the expression of functional AT2 receptors in tissues or cells derived from subjects genotyped as homozygous for either the G or A allele, which should provide a definitive answer. The fact that several independent groups have found association of this polymorphism with LVH and hypertension adds weight to the credibility of this polymorphism being functional. Again, the association is not clear cut. The A allele is associated with moderate increase in left ventricular mass in young mildly hypertensive males. However, male G allele carriers with above median sodium intake have similar left ventricular mass. In middle-aged patients with systemic hypertension, clinically relevant LVH in males is associated with carriers of the G allele. This is also related to a trend towards and association between the G allele and the presence of hypertension. An association of the G allele and premature CAD has also been established. In particular, there is increased prevalence of the G allele in males presenting with stenotic atherosclerosis requiring revascularisation. This adds weight to the in vivo animal studies which highlight a role for the AT2 receptor in the development and progression of atherosclerotic lesions. However, since at present it is not known for certain whether the G allele is associated with an increase or decrease in AT2 receptor expression, these data cannot be extrapolated to determine whether an increase in AT2 receptor expression in a human atherosclerotic lesion would be beneficial or harmful.

References


